Hyaluronan Contributes to Bronchiolitis Obliterans Syndrome and Stimulates Lung Allograft Rejection through Activation of Innate Immunity

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Abstract

Rationale: Although innate immunity is increasingly recognized to contribute to lung allograft rejection, the significance of endogenous innate ligands, such as hyaluronan (HA) fragments, in clinical or experimental lung transplantation is uncertain.

Objectives: To determine if HA is associated with clinical bronchiolitis obliterans syndrome (BOS) in lung transplant recipients, and evaluate the effect of low- or high-molecular-weight HA on experimental lung allograft rejection, including dependence on innate signaling pathways or effector cells.

Methods: HA concentrations were measured in bronchoalveolar lavage and plasma samples from lung recipients with or without established BOS. BOS and normal lung tissues were assessed for HA localization and expression of HA synthases. Murine orthotopic lung recipients with established tolerance were treated with low- or high-molecular-weight HA under varied experimental conditions, including Toll-like receptor (TLR) 2/4 and myeloid differentiation protein 88 deficiency and neutrophil depletion.

Measurements and Main Results: HA localized within areas of intraluminal small airways fibrosis in BOS lung tissue. Moreover, transcripts for HA synthase enzymes were significantly elevated in BOS versus normal lung tissues and both lavage fluid and plasma HA concentrations were increased in recipients with BOS. Treatment with low-molecular-weight HA abrogated tolerance in murine orthotopic lung recipients in a TLR2/4- and myeloid differentiation protein 88-dependent fashion and drove expansion of alloantigen-specific T lymphocytes. Additionally, TLR-dependent signals stimulated neutrophilia that promoted rejection. In contrast, high-molecular-weight HA attenuated basal allograft inflammation.

Conclusions: These data suggest that accumulation of HA could contribute to BOS by directly activating innate immune signaling pathways that promote allograft rejection and neutrophilia.

Keywords: lung transplantation; hyaluronan; bronchiolitis obliterans syndrome; innate immunity

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At a Glance Summary

Scientific Knowledge on the

Subject: Hyaluronan (HA) is an extracellular matrix component that has been demonstrated to activate innate immunity, regulate inflammation, and accumulate in fibrotic lung conditions. The role of HA in promoting clinical or experimental lung transplant rejection is not known.

What This Study Adds to the Field:

We demonstrate that HA accumulates in the lung fluid, tissue, and blood of lung transplant patients with bronchiolitis obliterans syndrome. In complementary studies using an experimental orthotopic lung transplant model, low-molecular-weight HA abrogates established tolerance, drives allograft rejection, and increases intra-graft neutrophil infiltration and activation in a Toll-like receptor–dependent fashion, thus providing insight into potential mechanisms by which HA may contribute to the development of bronchiolitis obliterans syndrome.

Lung transplantation is an established therapy for a range of end-stage pulmonary diseases. Long-term outcomes, however, remain limited primarily because of the onset of bronchiolitis obliterans syndrome (BOS) (1), which is characterized clinically by irreversible progressive airflow limitation and correlates histologically with findings of obliterative bronchiolitis (OB), or small airways fibrosis (2).

Although the mechanisms contributing to BOS development are not well understood, growing evidence implicates a range of host immune responses, including activation of innate and adaptive immunity (3). Consistent with this idea, we previously demonstrated that functional polymorphisms in Toll-like receptor (TLR) 4, a prototypic innate pattern recognition receptor, and its coreceptor CD14 modulate the risk for acute rejection or BOS after human lung transplantation (4, 5). Similarly, neutrophils, key innate effector cells, are highly associated with clinical BOS (6) and have been shown to function as a critical link between innate immunity and

alloimmunity in experimental lung transplantation (7).

Although pattern recognition receptors were first described to recognize molecular patterns on microbial pathogens, more recently they have also been noted to provide an internal mechanism by which to mount a response to injured self-tissue via recognition of endogenous damage-associated molecular patterns (DAMPs), such as heat shock proteins or hyaluronan (HA) fragments. HA is a principal extracellular matrix component that exists as a high-molecular-weight polymer (HMWHA) under homeostatic conditions and has been demonstrated to attenuate inflammation and protect from inflammatory injury (8–11). In response to tissue damage, however, low-molecular-weight HA (LMWHA) fragments are generated by the enzymatic action of hyaluronidases or synthesized *de novo* by local fibroblasts (8, 12). Recent findings suggest that these endogenous LMWHA fragments activate TLR signaling pathways to promote immune and inflammatory responses and could potentially contribute to allograft rejection (9, 13).

Based on these prior data, we developed the overall hypothesis that HA accumulates in clinical BOS and mechanistically contributes to the development of lung rejection by activating innate immunity and promoting neutrophil recruitment. To test this hypothesis, we first leveraged bronchoalveolar lavage (BAL), plasma, and lung tissue samples from carefully phenotyped lung recipients to determine if HA and HA synthase (HAS) transcripts are increased in clinical BOS. Next, we used the murine orthotopic lung transplant model to determine if LMWHA and HMWHA have differential effects on acute rejection in the setting of established tolerance. Finally, under these experimental conditions, we sought to determine if the effects of HA on the allograft are dependent on innate signaling through TLRs 2/4, their common adaptor molecule myeloid differentiation protein 88 (MyD88), or pulmonary neutrophilia.

Methods

Patient Population

Study cohorts were drawn from first, adult, single, or bilateral lung recipients at Duke University. Patients received similar post-

transplant management as described in the online supplement. BOS was defined according to international guidelines using serial pulmonary function tests (2). Studies were approved by the Duke University Internal Review Board (Pro00013378, Pro00007005, Pro00011676, and Pro0008725).

BAL Cohort

BAL samples were prospectively collected at the time of surveillance or clinically indicated bronchoscopy and processed as previously described (14). Table 1 describes the demographics of 114 recipients included in the cross-sectional BAL cohort. Fourteen of the 56 patients with BOS had an additional BAL sample obtained before BOS onset thus permitting longitudinal assessment within this subset. Only samples from bronchoscopies confirming freedom from concurrent acute rejection by transbronchial lung biopsy were included.

Plasma Cohort

Peripheral blood was collected from lung recipients with early or severe onset BOS (EO or SO BOS) (n = 12) and from long-term BOS-free patients (n = 26), identified as those remaining free from BOS for 5 or more years post-transplant (see Table E1 in the online supplement). EO BOS, BOS onset within 2 years of transplantation, and SO BOS, BOS onset at grade greater than or equal to two, have been recognized as aggressive BOS phenotypes associated with worse survival (15).

Tissue Cohort for Transcript and Histology Analysis

Lung tissue was procured from the lower lobe at the time of retransplantation or diagnostic thoracoscopy for BOS. Unused donor tissue, trimmed at the time of transplant because of size mismatch and demonstrated to be normal on histopathologic examination, was used as normal control lung tissue. Tissue RNA (BOS explant, n = 8, and donor control, n = 4) (see Table E2) was preserved, isolated, reverse transcribed, and Taqman assays for HAS1, HAS2, HAS3, and Col1 α 1 performed as detailed in the METHODS in the online supplement. Alcian blue histochemistry (BOS, n = 7) and immunofluorescence using antibodies to HA binding protein (BOS, n = 4, and donor control, n = 2) were used to assess HA

Table 1: Human Bronchoalveolar Lavage Cross-Sectional Cohort Demographics

Characteristic	BOS Free (n = 58)	BOS ≥ 1* (n = 56)
Age at transplant, yr [†]	53 (44,63)	51 (37,64)
White race	50 (86%)	48 (86%)
Female sex	26 (45%)	19 (34%)
Transplant indication		
COPD	22 (38%)	20 (36%)
Pulmonary fibrosis	17 (29%)	14 (25%)
Cystic fibrosis	10 (17%)	10 (18%)
Other	9 (16%)	12 (21%)
Bilateral lung transplant	56 (97%)	49 (88%)
Time to BAL collection, min ^{††}	44 (34,66)	74 (41,101)
Follow-up time, min ^{†§}	79 (67,100)	99 (61,138)
Normalized ACR score [†]	0.37 (0.19,0.54)	0.30 (0.22,0.50)

Definition of abbreviations: ACR = acute cellular rejection; BAL = bronchoalveolar lavage; BOS = bronchiolitis obliterans syndrome; COPD = chronic obstructive pulmonary disease.

*BOS 1, n = 20; BOS 2, n = 20; BOS 3, n = 16.

[†]Median (interquartile range).

^{††}Time from transplant to BAL sample collection.

[§]Time from transplant to death or November 16, 2011.

^{||}Normalized ACR score was defined as the sum of acute rejection grades divided by the number of assessable biopsies from transplant to the date of BAL sample collection. Acute rejection was identified as the presence of perivascular lymphocytic infiltration noted on transbronchial lung biopsy pathology and was graded according to 1996 International Society for Heart and Lung Transplantation criteria (28).

deposition and localization. Detailed methods are available in the online supplement.

HA Quantification

HA was measured in BAL and plasma using an HA sandwich ELISA (Echelon, Salt Lake City, UT), which detects a minimum HA size of 4.5 kD. Plasma samples were diluted at 1:10 as specified by the manufacturer. BAL samples were diluted at 1:5 and results adjusted appropriately. Samples falling below the standard curve were assigned an HA concentration of the lowest detectable value.

Murine Lung Transplantation, HA Airway Instillation, and Neutrophil Depletion

Murine lung transplantation was conducted and all graft recipients were treated with double costimulatory blockade to induce allograft acceptance as previously described (16–18). A total of 50, 100, or 200 µg of LMWHA (25–75 kD; Sigma, St. Louis, MO) or 200 µg of HMWHA (800–1,200 kD; Sigma) were administered in 50 µl of saline vehicle into the trachea of lung recipients with established tolerance on postoperative day (POD) 7, 14, and 21. To deplete neutrophils 250 µg of clone 1A8

antimouse Ly6G (BioXCell, West Lebanon, NH) was administered intravenously on POD 7, 14, and 21.

Mice

C57BL/6J (B6), MyD88^{−/−}, TLR2^{−/−}, TLR4^{−/−}, and Balb/cJ (Balb/c) mice were purchased from Jackson Laboratories (Bar Harbor, ME). MyD88^{−/−}, TLR2^{−/−}, and TLR4^{−/−} are on a B6 background. TLR2^{−/−}TLR4^{−/−} mice were generated by intercrossing TLR2^{−/−} and TLR4^{−/−} mice.

Murine Allograft Assessments

Hematoxylin and eosin sections were screened in double-blind fashion for the presentation of perivascular infiltrates and scored for A type rejection according to 2007 International Society for Heart and Lung Transplantation criteria (19). Lung tissue digests were examined for T-cell populations, BAL cytokines were analyzed, and alloantigen-specific CD4⁺ T-cell responses characterized as previously described (16, 18) and as described in the METHODS in the online supplement. Neutrophil reactive oxygen species (ROS) activity after vehicle, LMWHA (10 µg/ml) or HMWHA (10 µg/ml) stimulation, was measured by fluorescence-activated cell sorter analysis as detailed in the online supplement.

Statistical Analysis

Data are reported as mean ± standard error. Unpaired *t* test (with correction for unequal variances, as appropriate) was applied to assess the difference in mean BAL (cross-sectional data) and

Figure 1. Representative sections of lung tissue from a patient with advanced bronchiolitis obliterans syndrome stained with alcian blue (original magnification ×10). Representative airway affected by obliterative bronchiolitis. (A) Alcian blue staining localizes hyaluronan within fibrous tissue deposited in the airway lumen. (B) Pretreatment with hyaluronidase digestion abrogates staining within the airway lumen. Scant nonspecific staining is present within mucinous airway debris. Scale bar represents 100 µm. Representative images from two additional independent patients with bronchiolitis obliterans syndrome are included in Figure E7.

Figure 2. Representative sections of lung tissue from a patient with advanced bronchiolitis obliterans syndrome (original magnification $\times 10$) or normal control lung tissue (original magnification $\times 20$) assessed by immunofluorescence microscopy with antibodies against hyaluronan binding protein (green) and acetylated tubulin (red). (A) Representative affected airway within explanted lung tissue from a patient with bronchiolitis obliterans demonstrates hyaluronan localization within intraluminal fibrous tissue. Scale bar represents 100 μm . (B) Normal donor lung tissue with a normal small airway. Hyaluronan localizes to the extracellular matrix tissue around the normal airway but is not present within the airway lumen. Scale bar represents 100 μm . Representative images from two additional independent patients with bronchiolitis obliterans syndrome are included in Figure E8.

plasma HA levels between BOS free and BOS greater than or equal to one samples. Paired *t* test was applied to assess longitudinal differences in mean BAL HA levels between antecedent and BOS BAL samples. Transcript results were analyzed by nonparametric two-tailed *t* test (Mann-Whitney test). For

mouse experiments, significant differences were evaluated with an unpaired two-tailed Student *t* test. Analyses were performed using Graph Pad Prism 5.0 (GraphPad Software Inc., La Jolla, CA) and *P* values less than 0.05 were considered statistically significant.

Results

HA Deposits in Small Airways and HASs Are Up-regulated in BOS Lung Tissue

We first sought to determine whether HA deposits in small airways affected by OB after clinical lung transplantation. As shown

Figure 3. (A–D) Whole-lung transcripts for hyaluronan synthases 1 and 3 are significantly up-regulated in lung tissue from patients with end-stage bronchiolitis obliterans undergoing retransplantation ($n = 8$) as compared with normal control lung tissue ($n = 4$). * $P < 0.05$. BOS = bronchiolitis obliterans syndrome; HAS = hyaluronan synthase.

Figure 4. Hyaluronan levels are significantly elevated in bronchoalveolar lavage fluid from patients with, as compared with those without, bronchiolitis obliterans syndrome (BOS). (A) Cumulative cross-sectional bronchoalveolar lavage data represented as all BOS-free patients ($n = 58$) compared with all patients with BOS grades ≥ 1 ($n = 56$). (B) Cross-sectional bronchoalveolar lavage data represented as all BOS-free patients compared with those with BOS grades 1, 2, or 3, respectively. $*P < 0.05$. HA = hyaluronan.

in Figures 1 and 2, respectively, application of both immunohistochemistry with alcian blue and immunofluorescence with HA binding protein antibody localized HA deposition within areas of intraluminal fibrosis in small airways affected by pathologic OB. When BOS lung tissue was pretreated with hyaluronidase digestion, Alcian blue staining was eliminated (Figure 1B), indicating HA specificity. Notably, there was no evidence of submucosal expansion or HA deposition in the airways of normal donor control subjects (Figure 2B).

Because HA seemed to localize within OB lesions in the lung, we next sought to further determine whether pulmonary HA production is up-regulated in association with BOS. Enzymes important in HA synthesis were assessed by whole-lung transcript analysis for the three primary HAS isoforms: HAS1, HAS2, and HAS3 in explanted BOS versus normal donor lung tissue specimens. As demonstrated in Figures 3A and 3C, HAS1 and HAS3 mRNA expression were significantly increased in BOS-affected lung tissue when compared with that of normal donor control subjects (3.09-fold ± 0.79 vs. 0.86 ± 0.10 , $P = 0.048$ and 2.56-fold ± 0.47 vs. 1.04 ± 0.16 , $P = 0.004$, respectively). HAS2 and Col1a1 gene expression appeared increased in BOS versus normal donor lung tissue; however, this difference was not statistically significant (2.79-fold ± 0.79 vs. 1.12 ± 0.24 , $P = 0.28$ and 3.01-fold ± 1.07 vs. 1.03 ± 0.34 , $P = 0.37$, respectively) (Figures 3B and 3D).

HA Concentrations Are Increased in the BAL and Plasma in Clinical BOS

Because the above findings on pathology and transcript analysis supported increased pulmonary HA synthesis and deposition in patients with advanced BOS undergoing retransplantation, we next evaluated whether HA could be detected in the BAL fluid of patients with BOS even at less advanced stages of disease. Figures 4A and 4B outline BAL fluid HA ELISA results on a cross-sectional cohort of patients well characterized for BOS status at the time of BAL collection.

Notably, as shown in Figure 4A, HA levels were found to be significantly increased in patients with, as compared with those without, BOS (107.91 ± 13.8 ng/mL vs. 28.97 ± 4.48 ng/mL; $P < 0.0001$). Although HA increased significantly between BOS-free and BOS grade 1 status (28.97 ± 4.48 ng/mL vs. 82.62 ± 17.64 ng/mL; $P = 0.008$), further incremental increases correlating with advancing BOS grade were nonsignificant when compared with BOS1 (Figure 4B).

To further test the observed association between increased BAL HA levels and BOS, assess whether HA may be systemically detected, and evaluate the association of HA with extreme BOS phenotypes, we collected plasma samples from patients with EO or SO BOS and patients who had remained BOS free for at least 5 years. As indicated in Figure 5, HA levels were significantly elevated in the plasma of patients with EO or SO BOS as compared with those who enjoyed long-term freedom from BOS

(90.37 ± 20.97 ng/mL vs. 44.42 ± 9.88 ng/mL; $P = 0.03$).

Next, to permit evaluation of the longitudinal variation in BAL HA levels over time after lung transplantation, specifically, before and after the onset of BOS, we examined a subset of patients with BOS from our cross-sectional BAL cohort who had an available BAL sample procured before the time of BOS onset. As shown in Figure E1, within the same patient, HA concentrations increased significantly after the onset of BOS (mean increase 64.63 ng/mL; 95% confidence interval, 20.72–108.5; $P = 0.007$).

LMWHA Fragments Break Established Mouse Lung Allograft Tolerance

To directly assess if HA prevents lung allograft survival we transplanted Balb/c lungs into fully MHC-mismatched C57BL/6 (B6) recipients and treated with double costimulatory blockade immunosuppression (Figure 6). Using this approach we have previously shown the establishment of lung allograft acceptance for more than 100 days (18, 20). On POD 7, 14, and 21 endotoxin-free LMWHA fragments (see Figure E2) or saline vehicle control was instilled into the lung recipient trachea and allograft inflammation was assessed on POD 28. Compared with recipients that received saline vehicle, those receiving 200 μ g LMWHA had severe histologic evidence of acute cellular

Figure 5. Plasma hyaluronan levels are significantly increased in lung transplant recipients with early onset or severe onset bronchiolitis obliterans syndrome (BOS) compared with those remaining BOS free for 5 years or more. $*P < 0.05$. Early onset BOS and severe onset BOS were defined according to prior literature as the onset of BOS within 2 years of transplantation or as a decline in FEV₁ to $\leq 65\%$ of the post-transplant baseline at BOS onset, respectively (15). EO = early onset; HA = hyaluronan; SO = severe onset.

Figure 6. Model to analyze hyaluronan-mediated effects on established lung allograft tolerance. Balb/c lungs are transplanted into recipients on a B6 genetic background and treated with CTLA4Ig and CD40L (CD154) antibodies on postoperative day (POD) 1 and 2, respectively, to induce allograft tolerance. On POD 7, 14, and 21, 50, 100, or 200 μ g of low-molecular-weight hyaluronan, 200 μ g of high-molecular-weight hyaluronan, or saline vehicle are instilled into the trachea. Lung recipients are killed on POD 28 and assessed for allograft survival and inflammation. HA = hyaluronan.

vascular rejection with pronounced peribronchiolar inflammatory infiltrates (Figure 7A; see Figures E3A and E3B). Moreover, accumulation of both BAL HA and intragraft HAS mRNA was demonstrated in untreated, nonimmunosuppressed lung recipients, indicating the association of HA synthesis with allograft rejection (see Figure E4).

Analysis of T cells isolated from LMWHA-treated rejecting lung allograft tissue showed marked accumulation of IFN- γ ⁺ CD4⁺, IFN- γ ⁺ CD8⁺, and IL-17⁺ CD4⁺ cells and a significant reduction in intragraft Foxp3⁺ CD4⁺ T cells (Figure 7B; see Figure E3C). Similar to prior observations in rejecting human lung recipients (21), LMWHA treatment was also accompanied by α -tubulin and collagen

V-specific IL-17⁺ CD4⁺ T cell accumulation (see Figure E5).

LMWHA-mediated Lung Allograft Rejection Is Dependent on TLR Signaling

Prior observations have shown that HA fragments activate innate immune signaling pathways controlled by TLRs 2 and 4, and the TLR adaptor molecule MyD88 (9). To this end, we asked if TLR signaling-deficient recipients of Balb/c lungs demonstrate resistance to the inflammatory effects of LMWHA. Balb/c→TLR2^{-/-} TLR4^{-/-} and Balb/c→MyD88^{-/-} lung recipients with established tolerance were treated with LMWHA on POD 7, 14, and 21 and evaluated for allograft inflammation on POD 28.

As compared with wild-type B6 recipients, there was little evidence of vascular cellular rejection in TLR2^{-/-}TLR4^{-/-} or MyD88^{-/-} lung recipients (Figure 7A vs. Figure 8A). Notably, in LMWHA-treated TLR signaling-deficient lung recipients the percent abundance of intragraft IFN- γ ⁺ CD4⁺, IFN- γ ⁺ CD8⁺, and IL-17⁺ CD4⁺ T cells and Foxp3⁺ CD4⁺ T cells was similar to saline-treated wild-type B6 recipients (Figure 8B vs. Figure 7B). Furthermore, resistance to LMWHA-mediated cellular inflammation was associated with little change in the accumulation of alloantigen-specific IFN- γ ⁺ CD4⁺ and IL-17⁺ CD4⁺ T cells (Figure 8C) indicating that TLR2/4 and MyD88 signaling pathways drive alloimmune responses after LMWHA stimulation.

To further characterize TLR signaling-dependent inflammatory effects of LMWHA in lung recipients we assessed the BAL fluid concentration for IL-1 β , IL-6, and tumor necrosis factor (TNF)- α because these cytokines are well-established downstream transcriptional targets of TLR-MyD88 signaling pathways (Figure 8D). IL-6 and IL-1 β , and to a lesser extent TNF- α expression, were lower in the allografts of TLR2^{-/-}TLR4^{-/-} and MyD88^{-/-} lung recipients when compared with wild-type B6 recipients.

LMWHA Promotes Neutrophil Infiltration and Activation

The presence of high levels of graft neutrophils is often associated with lung rejection and recent reports have demonstrated that these innate cells directly promote alloantigen responses (7, 18). We next analyzed neutrophil graft infiltration in LMWHA-treated wild-type and TLR-signaling mutant lung recipients through counting neutrophils in the BAL. Wild-type B6 recipients treated with LMWHA, when compared with either saline-treated wild-type or LMWHA-treated TLR-signaling mutants, had significantly more neutrophils (Figure 9A). Also, in the BAL fluid we observed high concentrations of the chemokine KC (CXCL1), a mouse analog of IL-8 that promotes neutrophil tissue infiltration (Figure 9B) (22).

Because these data collectively suggested that neutrophils drive LMWHA-mediated acute rejection we next asked if reducing granulocyte numbers helps maintain established tolerance in these

Figure 7. Low-molecular-weight hyaluronan prevents established lung allograft tolerance in the context of murine orthotopic lung transplantation. Balb/c→B6 lung recipients with established tolerance received saline or low-molecular-weight hyaluronan on postoperative day 7, 14, and 21 and were evaluated on postoperative day 28 for acute cellular rejection by (A) hematoxylin and eosin histology (original magnification $\times 100$; inset, $\times 400$) and (B) percent abundance of indicated intragraft T-lymphocyte populations. Results are representative of at least four independent experiments. H&E = hematoxylin and eosin; LMWHA = low-molecular-weight hyaluronan.

Figure 8. Toll-like receptor signaling pathways control low-molecular-weight hyaluronan-mediated lung rejection. Balb/c→TLR2^{-/-}4^{-/-} and Balb/c→MyD88^{-/-} lung recipients with established tolerance received low-molecular-weight hyaluronan on postoperative day (POD) 7, 14, and 21 and assessed on POD 28 for rejection by (A) hematoxylin and eosin histology (original magnification ×100; inset, ×400) and (B) the percent abundance of indicated intragraft T-lymphocyte populations. Results are representative of four independent experiments. (C) POD 28 CD4⁺ T cells isolated from the spleens of either untransplanted (No LTx) B6 (wild-type) mice or indicated recipients of Balb/c lungs were stimulated with irradiated Balb/c splenocytes for 48 hours and assessed for IFN-γ or IL-17A recall expression. Results are representative of three independent experiments and are illustrated as mean cell number ± SE calculated from quadruplicate stimulations. (D) POD 28 bronchoalveolar lavage fluid cytokine concentrations from indicated recipients of Balb/c lungs. Data shown as mean concentration ± SE calculated from triplicate quantitation and are representative of four independent experiments. **P* < 0.05. H&E = hematoxylin and eosin; LMWHA = low-molecular-weight hyaluronan; LTx = lung transplant; MyD88 = myeloid differentiation protein 88; TLR = Toll-like receptor; TNF = tumor necrosis factor; WT = wild-type.

recipients. Accordingly, Balb/c→B6 lung recipients with established tolerance were treated with LMWHA along with either control immunoglobulin or neutrophil-depleting Abs on POD 7, 14, and 21 and assessed for allograft tolerance on POD 28. Lung recipients treated with neutrophil-depleting Abs maintained tolerance, whereas control immunoglobulin-treated recipients acutely rejected their lungs

(Figure 9C). Resistance to acute rejection was also associated with a lower percent abundance of intragraft IFN-γ⁺ CD4⁺, IFN-γ⁺ CD8⁺, and IL-17⁺ CD4⁺ T cells (Figure 9D).

Given that neutrophils express TLR2 and 4 (23) we analyzed ROS production to determine if LMWHA fragments directly promote neutrophil activation (Figure 9E). LMWHA treatment sharply increased neutrophil ROS production in a

predominantly TLR2/4/MyD88-dependent manner.

HMWHA Fragments Inhibit Allograft Inflammation in Tolerant Lung Recipients

We next determined the effects of HMWHA fragments on lung transplants. Hence, we treated Balb/c→B6 lung recipients with established tolerance on POD 7, 14, and 21 with HMWHA and assessed allograft tolerance on POD 28. Unlike lung recipients that received LMWHA, HMWHA-treated lung recipients maintained tolerance (Figure 10A vs. Figure 7A; see Figure E6). Consistent with this observation the relative abundance of intragraft IFN-γ⁺ CD4⁺, IFN-γ⁺ CD8⁺, and IL-17⁺ CD4⁺ T cells were comparable with saline-treated lung recipients (Figure 10B vs. Figure 7B). However, there were significantly fewer numbers of these T-lymphocyte populations in the lung allografts of HMWHA-treated recipients (Figure 10C). This was also true for FoxP3⁺CD4⁺ T-cell numbers, except the ratio of these cells to IFN-γ⁺ CD8⁺ T cells remained similar to saline-treated lung recipients (Figure 10D). Because these data suggested that HMWHA treatment inhibited inflammation we next assessed the BAL for neutrophil accumulation and expression of inflammatory mediators. HMWHA-treated lung recipients had significantly fewer airway neutrophils (Figure 10E), lower levels of BAL IL-1β, IL-6, and TNF-α when compared with saline-treated recipients (Figure 10F). Consistent with this diminished inflammatory cytokine production, we additionally observed that HMWHA pretreatment suppressed LMWHA-mediated neutrophil ROS activity (Figure 10G).

Discussion

Although HA has been shown to regulate innate inflammatory responses (9, 24, 25), its contribution to lung allograft rejection has not yet been well characterized either clinically or experimentally. In a small cross-sectional analysis, we previously found that HA was significantly increased in the lung fluid of patients with BOS (13). In the current study, we expand the scope of our analysis to consider a longitudinal assessment of lung fluid HA and evaluation

Figure 9. Low-molecular-weight hyaluronan (LMWHA)-triggered lung allograft rejection is dependent on neutrophilia. Postoperative day (POD) 28 bronchoalveolar lavage (A) neutrophil numbers and (B) cytokine KC concentrations from indicated lung recipients of Balb/c lungs. Data shown in A are mean neutrophils numbers $\pm$ SE and in B mean concentration $\pm$ SE calculated from triplicate quantitation. Both results are representative of four independent experiments. Balb/c $\rightarrow$ B6 lung recipients with established tolerance were treated with LMWHA along with either Ly6G Ab (clone 1A8) or control immunoglobulin on POD 7, 14, and 21 and were evaluated on POD 28 for (C) efficacy of neutrophil depletion and hematoxylin and eosin histology (original magnification $\times 100$; inset, $\times 400$), and (D) percent abundance of indicated intragraft T-lymphocyte populations. (E) B6 wild-type neutrophils stimulated with saline (black) or LMWHA (red). TLR2^{-/-} or MyD88^{-/-} neutrophils stimulated with LMWHA (green and blue, respectively). Ten minutes later reactive oxygen species production analysis was conducted by fluorescence-activated cell sorter analysis using the fluorogenic probe DHR123. Results are representative of four independent experiments. * $P < 0.05$. BAL = bronchoalveolar lavage; DHR123 = dihydrorhodamine 123; H&E = hematoxylin and eosin; MyD88 = myeloid differentiation protein 88; TLR = Toll-like receptor; WT = wild-type.

of HA expression in the blood. This extended analysis demonstrates that HA is increased in the lung fluid and blood of patients with, as compared with those without, BOS and that increases in BAL HA occur in association with the development of BOS. Moreover, we make the novel observations that transcripts for HAS1 and HAS3 are up-regulated in BOS lung tissue and that HA deposits within small airways affected by OB.

To better understand these clinical observations, we leveraged the murine orthotopic lung transplant model to directly assess the mechanisms by which HA influences lung rejection *in vivo* and better delineate the role of HA fragment size in the context of alloimmunity. Our experimental results demonstrate that HA fragments of low, but not high, molecular weight stimulate acute allograft rejection in lung recipients with established tolerance in

a TLR2/4- and MyD88-dependent manner. Notably, the rejection invoked by LMWHA in our model was accompanied not only by an increase in IFN- γ ⁺CD4⁺ and IFN- γ ⁺CD8⁺ T-cell populations, but also by intragraft IL-17⁺CD4⁺ T-cell accumulation. These results are consistent with those in a single prior report evaluating HA in experimental skin transplantation, which demonstrated that, in a largely TLR4-dependent manner, LMWHA fragments promote dendritic cell maturation, expand allogeneic CD4⁺ T cells, and direct a Th1 response (13). To the best of our knowledge, however, ours is the first report of HA driving development of a Th17 response through a TLR-dependent mechanism. These results corroborate the importance of Th17 cells in lung rejection and clinical BOS (26). Additionally, we observed that LMWHA treatment increased IL-6 expression through engagement of TLR-MyD88 pathways likely skewing the alloresponse in a Th1 and Th17, rather than T-regulatory, direction. Together, these data suggest that LMWHA-mediated allograft rejection is triggered through TLR-MyD88 signaling pathways that disrupt critical intragraft regulatory mechanisms and promote differentiation of effector T-cell populations.

Our results provide insight into the specific importance of TLR signaling in recipient-derived cells, because inhibition of recipient TLR-MyD88 pathways protected against the rejection response induced by LMWHA. Interestingly, these experimental results parallel our prior clinical studies, which demonstrated recipient but not donor TLR4 polymorphisms that blunt innate immunity decreased the risk for acute rejection (4, 5). Although we did not formally test the role of TLR signaling with respect to HMWHA, Jiang and coworkers (9) previously demonstrated that in the context of experimental bleomycin exposure HMWHA attenuated acute lung injury pathology and inhibited epithelial cell apoptosis in a TLR2/4 and MyD88 fashion. Interestingly, we observed similar beneficial effects of HMWHA within our experimental lung transplant model. As such, it is plausible that these observed effects may have been conferred through TLRs expressed on lung structural cells, although further experiments are necessary to test this idea.

Recent data have demonstrated the importance of activated neutrophils in

Figure 10. High-molecular-weight hyaluronan (HMWHA) inhibits inflammation in lung recipients with established tolerance. Balb/c→B6 lung recipients with established tolerance received HMWHA on postoperative day (POD) 7, 14, 21 and were evaluated on POD 28 for rejection by (A) hematoxylin and eosin histology (original magnification $\times 100$; inset, $\times 400$) and the (B) percent abundance of indicated intra-graft T-lymphocyte populations. (C) B6 recipients of Balb/c lungs were treated as shown, analyzed by a fluorescence-activated cell sorter for indicated T-cell phenotype and quantified per allograft. Data are expressed as mean number per graft $\pm$ SE calculated from at least three lung transplants. (D) Ratio of the number of intra-graft IFN- γ ⁺ CD8⁺ T cells (CTL) to the number of intra-graft FoxP3⁺ CD4⁺ T cells. (E) POD 28 bronchoalveolar lavage neutrophil numbers and fluid cytokine concentrations of KC from resting B6 mice or B6 recipients of Balb/c lungs treated as indicated. Data shown in D are mean neutrophils numbers or ratio $\pm$ SE and in E mean concentration $\pm$ SE calculated from triplicate quantitation. (F) POD 28 bronchoalveolar lavage fluid concentrations of indicated cytokines from resting B6 mice or B6 recipients of Balb/c lungs treated as shown. Data represented as mean concentration $\pm$ SE calculated from triplicate quantitation. (G) Neutrophils stimulated with either vehicle (black), HMWHA (blue), low-molecular-weight hyaluronan (red), or HMWHA and then low-molecular-weight hyaluronan (green) were evaluated for reactive oxygen species production. Results are representative of at least three independent experiments (A, B, E, F, and G). * $P < 0.05$. H&E = hematoxylin and eosin; LMWHA = low-molecular-weight hyaluronan; LTx = lung transplant; TNF = tumor necrosis factor.

mediating experimental lung transplant rejection either by enhancing antigen presentation or by directly promoting

costimulation of intra-graft T cells (7, 18). Importantly, neutrophilia was required for LMWHA-mediated acute rejection

indicating that granulocytes play a critical effector role in HA-induced alloimmunity. Similarly, we observed high concentrations of the chemokine KC, a mouse analog of human IL-8 that promotes neutrophil tissue infiltration (22), in LMWHA-treated as opposed to saline-treated wild-type B6 recipients. Consistent with these observations, LMWHA was previously demonstrated to increase human bronchial epithelial cell IL-8 expression in a dose-dependent manner (12). Beyond neutrophil recruitment, we additionally observed that LMWHA increased neutrophil ROS activity in a TLR signaling-dependent manner, suggesting that LMWHA promotes graft tissue damage by TLR-mediated neutrophil activation.

Collectively, our results support a novel conceptual paradigm of BOS development and progression in which innate immune activation by endogenous DAMPs directly regulates the host alloimmune response to the transplanted lung. In this framework, allograft tolerance is established after clinical lung transplantation through commonly applied induction and maintenance immunosuppression; however, intervening clinical events, such as infection or aspiration, injure the airway epithelium and generate LMWHA fragments or other DAMPs. Failed clearance of LMWHA by-products or a sustained cellular phenotypic change that leads to increased basal LMWHA synthesis promotes prolonged Th1 and Th17 alloimmunity and diminishes T-regulatory cells, thus precipitating acute rejection. Infiltrating neutrophils and their derived products subsequently propagate local tissue injury and promote fibrosis. This conceptual model provides a cohesive underlying mechanism for many of the previous independent reports of BAL cellular and protein constituents associated with clinical BOS (6). For example, although the reasons for BAL neutrophilia in BOS are not well understood, our results suggest that this cellular shift may be a direct consequence of innate activation by DAMPs.

Despite the complementary clinical and experimental results, our study has several limitations. First, is the inability to directly assess the molecular weight of the HA present in the human BAL or plasma samples. Given the inherently dilute nature of these fluids, quantities sufficient to allow accurate quantification of HA fragment size were not available, although we made

unsuccessful attempts with agarose gel electrophoresis. Despite this limitation our clinical data clearly demonstrate significant up-regulation of HAS1 and HAS3 transcripts in BOS lung tissue. Because HAS3, in particular, is known to synthesize HA of lower molecular weight (27), our results would be consistent with accumulation of at least some LMWHA within patients with BOS. Second, our experimental model is one of acute rejection rather than BOS. For reasons that remain uncertain it has proved rather difficult to experimentally model classic pathologic OB lesions in mice. Interestingly, in the one study that did describe OB lesions using a minor mismatch model (26), Th17 cells played a central role in the development of OB

lending additional significance to our findings regarding the importance of LMWHA in driving a Th17 phenotype. Finally, although these clinical data are robust, further studies are needed to define associations between longitudinal changes in HA among lung recipients and the spectrum of airway histology that precedes OB, including lymphocytic bronchiolitis.

In conclusion we demonstrate that HA accumulates in the tissue, lung fluid, and blood of lung transplant recipients with BOS. In its low-molecular-weight form, HA abrogates tolerance and promotes rejection in experimental lung transplantation through a mechanism dependent on innate immunity and neutrophils. These novel observations offer

insights into a broader conceptual paradigm of BOS development and progression and furthermore suggest novel treatment targets to prevent BOS. Ultimately, continued refinement in the understanding of the link between allograft injury, endogenous innate ligands, and adaptive immunity is necessary to fully develop new therapies that significantly improve long-term patient outcomes. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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References

- Christie JD, Edwards LB, Kucheryavaya AY, Benden C, Dipchand AI, Dobbels F, Kirk R, Rahmel AO, Stehlik J, Hertz MI; International Society of Heart and Lung Transplantation. The Registry of the International Society for Heart and Lung Transplantation: 29th adult lung and heart-lung transplant report-2012. *J Heart Lung Transplant* 2012;31:1073–1086.
- Estenne M, Maurer JR, Boehler A, Egan JJ, Frost A, Hertz M, Mallory GB Jr, Snell GI, Yousem S. Bronchiolitis obliterans syndrome 2001: an update of the diagnostic criteria. *J Heart Lung Transplant* 2002;21:297–310.
- Todd JL, Palmer SM. Bronchiolitis obliterans syndrome: the final frontier for lung transplantation. *Chest* 2011;140:502–508.
- Palmer SM, Klimeck W, Yu L, Reinsmoen NL, Snyder LD, Ganous TM, Burch L, Schwartz DA. Genetic regulation of rejection and survival following human lung transplantation by the innate immune receptor CD14. *Am J Transplant* 2007;7:693–699.
- Palmer SM, Burch LH, Trindade AJ, Davis RD, Herczyk WF, Reinsmoen NL, Schwartz DA. Innate immunity influences long-term outcomes after human lung transplant. *Am J Respir Crit Care Med* 2005;171:780–785.
- Kennedy VE, Todd JL, Palmer SM. Bronchoalveolar lavage as a tool to predict, diagnose and understand bronchiolitis obliterans syndrome. *Am J Transplant* 2013;13:552–561.
- Kreisel D, Sugimoto S, Zhu J, Nava R, Li W, Okazaki M, Yamamoto S, Ibrahim M, Huang HJ, Toth KA, et al. Emergency granulopoiesis promotes neutrophil-dendritic cell encounters that prevent mouse lung allograft acceptance. *Blood* 2011;118:6172–6182.
- Jiang D, Liang J, Noble PW. Hyaluronan as an immune regulator in human diseases. *Physiol Rev* 2011;91:221–264.
- Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, Prestwich GD, Mascarenhas MM, Garg HG, Quinn DA, et al. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nat Med* 2005;11:1173–1179.
- Campo GM, Avenoso A, D'Ascola A, Scuruchi M, Prestipino V, Nastasi G, Calatroni A, Campo S. The inhibition of hyaluronan degradation reduced pro-inflammatory cytokines in mouse synovial fibroblasts subjected to collagen-induced arthritis. *J Cell Biochem* 2012;113:1852–1867.
- Shimizu M, Yasuda T, Nakagawa T, Yamashita E, Julovi SM, Hiramitsu T, Nakamura T. Hyaluronan inhibits matrix metalloproteinase-1 production by rheumatoid synovial fibroblasts stimulated by proinflammatory cytokines. *J Rheumatol* 2003;30:1164–1172.
- Mascarenhas MM, Day RM, Ochoa CD, Choi WI, Yu L, Ouyang B, Garg HG, Hales CA, Quinn DA. Low molecular weight hyaluronan from stretched lung enhances interleukin-8 expression. *Am J Respir Cell Mol Biol* 2004;30:51–60.
- Tesar BM, Jiang D, Liang J, Palmer SM, Noble PW, Goldstein DR. The role of hyaluronan degradation products as innate alloimmune agonists. *Am J Transplant* 2006;6:2622–2635.
- Kelly FL, Kennedy VE, Jain R, Sindhvani NS, Finlen Copeland CA, Snyder LD, Eu JP, Meltzer EB, Brockway BL, Pavlisko E, et al. Epithelial clara cell injury occurs in bronchiolitis obliterans syndrome after human lung transplantation. *Am J Transplant* 2012;12:3076–3084.
- Finlen Copeland CA, Snyder LD, Zaas DW, Turbyfill WJ, Davis WA, Palmer SM. Survival after bronchiolitis obliterans syndrome among bilateral lung transplant recipients. *Am J Respir Crit Care Med* 2010;182:784–789.
- Okazaki M, Krupnick AS, Kornfeld CG, Lai JM, Ritter JH, Richardson SB, Huang HJ, Das NA, Patterson GA, Gelman AE, et al. A mouse model of orthotopic vascularized aerated lung transplantation. *Am J Transplant* 2007;7:1672–1679.
- Krupnick AS, Lin X, Li W, Okazaki M, Lai J, Sugimoto S, Richardson SB, Kornfeld CG, Garbow JR, Patterson GA, et al. Orthotopic mouse lung transplantation as experimental methodology to study transplant and tumor biology. *Nat Protoc* 2009;4:86–93.
- Yamamoto S, Nava RG, Zhu J, Huang HJ, Ibrahim M, Mohanakumar T, Miller MJ, Krupnick AS, Kreisel D, Gelman AE. Cutting edge: *Pseudomonas aeruginosa* abolishes established lung transplant tolerance by stimulating B7 expression on neutrophils. *J Immunol* 2012;189:4221–4225.
- Stewart S, Fishbein MC, Snell GI, Berry GJ, Boehler A, Burke MM, Glanville A, Gould FK, Magro C, Marboe CC, et al. Revision of the 1996 working formulation for the standardization of nomenclature in the diagnosis of lung rejection. *J Heart Lung Transplant* 2007;26:1229–1242.
- Li W, Bribriescio AC, Nava RG, Brescia AA, Ibricevic A, Spahn JH, Brody SL, Ritter JH, Gelman AE, Krupnick AS, et al. Lung transplant acceptance is facilitated by early events in the graft and is associated with lymphoid neogenesis. *Mucosal Immunol* 2012;5:544–554.
- Saini D, Weber J, Ramachandran S, Phelan D, Tiriveedhi V, Liu M, Steward N, Aloush A, Hachem R, Trulock E, et al. Alloimmunity-induced autoimmunity as a potential mechanism in the pathogenesis of chronic rejection of human lung allografts. *J Heart Lung Transplant* 2011;30:624–631.

22. Huang S, Paulauskis JD, Godleski JJ, Kobzik L. Expression of macrophage inflammatory protein-2 and KC mRNA in pulmonary inflammation. *Am J Pathol* 1992;141:981–988.
23. Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. *Blood* 2003;102:2660–2669.
24. McKee CM, Penno MB, Cowman M, Burdick MD, Strieter RM, Bao C, Noble PW. Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. *J Clin Invest* 1996;98:2403–2413.
25. Noble PW, McKee CM, Cowman M, Shin HS. Hyaluronan fragments activate an NF-kappa B/I-kappa B alpha autoregulatory loop in murine macrophages. *J Exp Med* 1996; 183:2373–2378.
26. Fan L, Benson HL, Vittal R, Mickler EA, Presson R, Fisher AJ, Cummings OW, Heidler KM, Keller MR, Burlingham WJ, *et al*. Neutralizing IL-17 prevents obliterative bronchiolitis in murine orthotopic lung transplantation. *Am J Transplant* 2011;11: 911–922.
27. Itano N, Kimata K. Mammalian hyaluronan synthases. *IUBMB Life* 2002;54:195–199.
28. Yousem SA, Berry GJ, Cagle PT, Chamberlain D, Husain AN, Hruban RH, Marchevsky A, Ohori NP, Ritter J, Stewart S, *et al*. Revision of the 1990 working formulation for the classification of pulmonary allograft rejection: Lung Rejection Study Group. *J Heart Lung Transplant* 1996;15: 1–15.